Long-Term Effects on the Female Mouse Genital Tract Associated with Prenatal Exposure to Diethylstilbestrol

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ABSTRACT

The association between treatment of pregnant women with diethylstilbestrol (DES) and reproductive tract abnormalities in their female offspring is well known. Reports of comparable in utero exposure in animals are few. In this paper, pregnant outbred mice were treated s.c. with daily doses of DES ranging from 0.01 to 100 µg/kg on Days 9 to 16 of gestation. This period corresponds with major organogenesis of the reproductive tract in the mouse. Additional groups of pregnant mice were treated during the same time interval with 100-µg/kg doses of either dimethylstilbestrol (DMS), a weakly estrogenic structural analog of DES, or 17β-estradiol, a potent thyroidal estrogen. When female offspring of mice gestationally exposed to DES were sacrificed at 12 to 18 months of age, lesions were found throughout their reproductive tracts. The vagina was characterized by excess keratinization and female hypoplasia, and, at the dose of 100 µg/kg, 5 of 20 mice had epidermoid tumors of the vagina; in one of the 35 mice derived from mothers treated with DES (10 µg/kg), vaginal adenocarcinoma was observed. The cervix in the DES-exposed offspring was enlarged, even though the size of its lumen was not different from that in the controls. Stromal stimulation of the cervix was apparent, and a low incidence of benign (leiomyoma, papilloma) as well as malignant (stromal cell sarcoma, leiomyosarcoma) tumors were seen. There was evidence of stimulation in the epithelium, and stroma of the uterus and cystic endometrial hyperplasia was common; a low incidence of benign (leiomyomas) and malignant (adenocarcinoma, stromal cell sarcoma) tumors was observed. The ovaries of prenatally DES-treated females were cystic more often than those of the corresponding controls; at the highest dose, three ovarian tumors were noted, and the fallopian tubes were inflamed and congenitally malformed. Genital tract tumors were not seen in 85 control females or in the 12 or 10 females exposed prenatally to estradiol or DMS, respectively. In fact, in 12- to 13-month-old females derived from mice exposed during pregnancy to DES (100 µg/kg), common findings, which were absent or in very low incidence in control, estradiol-treated, or DMS-treated mice, included: vaginal concretions and excess keratinization; cervical enlargement; uterine squamous metaplasia and cystic endometrial hyperplasia; and oviductal malformations. The differences in effects among DES, estradiol, and DMS may be linked to differences in relative bioavailability of the compounds to the fetal target tissue. The results presented suggest a role for different embryonic rudiments as well as for tissue components in the observed long-term effects of gestational exposure to DES on the female reproductive tract. Moreover, such prenatal studies with DES in mice may be helpful in understanding the role of estrogens in the functional development of the genital tract and may ultimately provide a useful experimental model.

INTRODUCTION

Steroid hormones are known to be important in the development of the mammalian genital tract. In early fetal life, testicular hormones determine the differentiation of typically male sexual structures. In females, the development of the accessory sex organs is apparently not dependent on fetal hormones (17). The role of estrogenic hormones in the normal differentiation of the fetal reproductive tract is not completely known. Since the 1971 report of Herbst et al. (16) on the association between treatment of pregnant women with the synthetic estrogen, DES,2 and vaginal clear-cell adenocarcinoma in female offspring, interest has increased in the long-term consequences of exposure to estrogenic substances during critical periods of development.

Studies in other laboratories (3, 10, 32) have demonstrated that administration of estrogens to mice in the neonatal period is associated with long-term effects in the genital tract including preneoplastic and neoplastic changes in the vaginal and cervical epithelium. Such observations made in neonatally treated mice should have significance to prenatally exposed humans, since some of the same processes of later cytodiifferentiation of the cervicovaginal epithelium (formation of pseudostratified columnar to squamous epithelium) occur during early fetal life in humans and in the first week of life in mice (11). However, other processes, such as organogenesis of the genital tract and early differentiation of both the cervicovaginal and uterine epithelium, occur prenatally in both species. Thus, comparisons of results from prenatal, as well as neonatal, experiments with mice may provide useful information for the clinical situation.

The present report describes the long-term effects of gestational administration of DES to mice during the critical period of reproductive tract organogenesis and early cell differentiation of these structures. Moreover, comparisons to a poorly estrogenic structural analog of DES (DMS) and to a steroidal estrogen (17β-estradiol) are made. Results obtained in a small number of the animals included in the present report have been published previously in preliminary form (21).

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2 The abbreviations used are: DES, diethylstilbestrol; DMS, dimethylstilbestrol; AFP, α-fetoprotein.
MATERIALS AND METHODS

Animals. Outbred CD-1 mice were purchased as timed-pregnant from Charles River Breeding Laboratories, Wilmington, Mass., or bred to male mice of the same strain at our animal quarters. Detection of a vaginal plug was considered Day 0 of pregnancy. Pregnant mice were housed individually in a temperature-controlled room (21–22°C) with 14-hr light and 10-hr dark periods and were provided with synthetic bedding, fresh water, and Purina laboratory mouse chow ad libitum.

Chemicals. DES and 17β-estradiol were purchased from Sigma Chemical Company, St. Louis, Mo. Purity was checked by thin-layer chromatography, high-pressure liquid chromatography, and gas chromatography-mass spectrometry. DMS was a gift from Dr. John Katzenellenbogen, Department of Chemistry, University of Illinois, Urbana, Ill.

Animal Experiments. Pregnant mice were treated s.c. with the appropriate compound on Days 9 through 16, inclusive, of gestation. The daily doses of DES used were 0.01, 1, 2.5, 5, 10, and 100 μg/kg of maternal body weight; these groups of animals are referred to in this report as DES-0.01, DES-1, DES-2.5, DES-5, DES-10, and DES-100, respectively. DES, estradiol, and DMS were dissolved in corn oil; in the case of estradiol, the compound was first dissolved in ethanol and then mixed with corn oil (<1% ethanol). The total volume of corn oil administered to both drug-treated and control animals was 0.01 ml/g of maternal body weight.

At birth, the litters were examined, and the litter sizes were reduced to 8, where appropriate; at 25 days of age, the mice were weaned and housed in groups of 2 to 5. At the ages indicated in Table 1, the mice were sacrificed by decapitation. Gross observations of any unusual external features were recorded. The abdominal cavity was then rapidly opened, and observations were made on all internal organs with particular attention to the anatomical relationships of the genital tract and surrounding structures. The entire vagina, cervix, uterus, and bladder were carefully removed and pinned to cardboard to preserve the orientation of the entire organ system. All tissues were fixed in 10% neutral buffered formalin except the ovaries and oviducts which were fixed in Bouin’s solution for 24 hr and then transferred to 70% ethanol. After thorough examination of the abdominal cavity and removal of any abnormal lymph nodes, the thoracic cavity was opened, and the heart, lungs, and trachea were dissected out intact. Any gross lesions such as mammary tumors and any enlarged lymph nodes were also removed.

After fixation, all organs were dehydrated and then embedded in paraffin, sectioned at 6 μm and stained with hematoxylin and eosin. The vagina and cervix were cut in the midsagittal plane. Both halves were embedded in the same block and were serially sectioned sagittally for 10 sections starting at the sagittal midline. If a microscopic lesion was observed, additional serial sections were made to include the entire area of pathological change. In some cases, the paraffin sections were stained by the periodic acid-Schiff reaction, Wilder’s reticulum stain, Mayer’s mucicarmine, or oil red O. Cervical size determinations were made on a representative section from 3 cross-sections through the uterine cervix; the size of the cervical lumen was determined by measuring the largest distance from the basement membrane on one side of the cervix to the other on each cross-section. Statistical significance was determined by the 2-sample t test. In one case, the paraffin block was deparaffinized, reembedded in plastic, and cut into thin sections for evaluation on a Phillips EM-300 electron microscope.

Embryonic Tissue Grafts. Embryonic tissue explants were obtained from fetuses of pregnant CD-1 females at 14 days of gestation. Control tissue explants were removed from fetuses of females receiving no treatment, and treated tissue explants were from the fetuses of females receiving DES (100 μg/kg maternal weight) on Days 9 through 14 of gestation. Ovarian tissue was dissected from female embryos, with the aid of a dissecting microscope, in Petri dishes containing Earle’s phosphate-buffered saline (pH 7.2). Tissues were then grafted under the kidney capsules of 8-week-old female CD-1 hosts which were ovariectomized at the time of grafting. Nineteen days after grafting, the hosts were sacrificed, and the grafts were removed; the grafts were fixed in Bouin’s fixative, embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin.

RESULTS

Prevalence of Changes. Prenatal exposure to DES was associated with a dose-related increase in genital tract abnormalities in 12- to 18-month-old female offspring (Table 2). The prevalence of histological abnormalities of the vagina ranged from 1% among control mice to 90% for the group exposed prenatally to DES-100. The corresponding value for the cervix of control mice was 1%, while that for the cervix of DES-100 mice was 60%. The uterus was also affected by gestational exposure to DES, since only 8% of control mice had uterine abnormalities compared to 85% of the DES-100 offspring. The abnormalities in control animals appeared at a later age (18 months) than they did in DES-treated animals and were consistent with the usual age-related changes; in DES-treated animals, abnormalities could be found at 12 to 13 months of age.

The number of genital tract tumors was low in all the mice treated with DES at a dose less than DES-100. The combined prevalence of tumors of the vagina, cervix, and uterus ranged from 9.1% (2 of 22) in DES-5 mice to 4.8% (1 of 21) in DES-0.01 mice. The corresponding value for control female offspring was 0% (0 of 85). In females treated with DES-100 in utero, 35% had genital tract tumors. Moreover, in the 3 highest dose groups, tumors were observed in animals sacrificed at 12 or 13 months of age.

The occurrence of nongenital lesions did not seem to be significantly different in control or treated groups. In fact, when
data on mammary lesions were compiled from all of the 12- to 18-month-old mice which had died or had been sacrificed and examined in our laboratory (including those in Table 1), the prevalence of extragenital lesions was not significantly different between treatment groups.

<table>
<thead>
<tr>
<th>DES dose (μg/kg/day)</th>
<th>Vaginal Abnormalities</th>
<th>Cervical Abnormalities</th>
<th>Uterine Abnormalities</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1/85 excess keratinization (18)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1/85 squamous metaplasia (18)</td>
<td>6/85 cystic endometrial hyperplasia (18)</td>
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<tr>
<td>0.01</td>
<td>1/21 excess keratinization (13)</td>
<td>1/21 edematous squamous metaplasia (13)</td>
<td>1/21 leiomyoma (15)</td>
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<tr>
<td>1</td>
<td>0/19</td>
<td>2/19 cystic glands (13)</td>
<td>1/19 squamous metaplasia (18)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1/19 papilloma (18)</td>
<td>1/19 squamous metaplasia (18)</td>
</tr>
<tr>
<td>2.5</td>
<td>1/17 excess keratinization (17)</td>
<td>1/17 stromal edema (17)</td>
<td>8/17 cystic endometrial hyperplasia (17, 18)</td>
</tr>
<tr>
<td>5</td>
<td>2/22 excess keratinization (18)</td>
<td>1/22 keratinized cysts (18)</td>
<td>9/22 cystic endometrial hyperplasia (17, 18)</td>
</tr>
<tr>
<td>10</td>
<td>2/35 excess keratinization (18)</td>
<td>1/35 hyalinization (18)</td>
<td>22/35 cystic endometrial hyperplasia (12, 13, 17)</td>
</tr>
<tr>
<td></td>
<td>1/35 hyalinization (18)</td>
<td>5/35 stromal edema (12, 13)</td>
<td>1/35 hypoplastic with squamous metaplasia (18)</td>
</tr>
<tr>
<td></td>
<td>1/35 edema (13)</td>
<td>3/35 squamous metaplasia (13, 18)</td>
<td>3/35 pyometra (12, 13)</td>
</tr>
<tr>
<td></td>
<td>5/35 urethral glands and opening (12, 13)</td>
<td>1/35 stromal hyperplasia (18)</td>
<td>3/35 squamous metaplasia (13, 17)</td>
</tr>
<tr>
<td></td>
<td>1/35 basal cell hyperplasia (13)</td>
<td>1/35 epithelial atypia (18)</td>
<td>1/35 leiomyoma (13)</td>
</tr>
<tr>
<td>100</td>
<td>6/20 excess keratinization (12, 13)</td>
<td>12/20 stromal edema (12, 13)</td>
<td>12/20 cystic endometrial hyperplasia (12, 13, 14, 17)</td>
</tr>
<tr>
<td></td>
<td>1/20 hyalinization (13)</td>
<td>5/20 squamous metaplasia (12, 13)</td>
<td>6/20 hypoplastic (12, 13)</td>
</tr>
<tr>
<td></td>
<td>2/20 edema (13)</td>
<td>2/20 hypoplastic (12, 13)</td>
<td>1/20 hyaline stroma (12)</td>
</tr>
<tr>
<td></td>
<td>2/20 hypoplastic (12, 13)</td>
<td>1/20 erosion (13)</td>
<td>6/20 squamous metaplasia (12, 13)</td>
</tr>
<tr>
<td></td>
<td>7/20 urethral opening or glands (12, 13, 17)</td>
<td>1/20 leiomyosarcoma (13)</td>
<td>3/20 pyometra (12, 13, 17)</td>
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<td></td>
<td>5/20 epidermoid tumor (12, 13)</td>
<td>1/20 adenocarcinoma (13)</td>
<td>1/20 adenocarcinoma (13)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Numbers in parentheses, age at sacrifice as noted in Table 1.
<sup>b</sup> Metastases found in uterine horns, fallopian tubes, and liver.

sections from DES-treated females and, at higher doses, were often cystic (Fig. 1). For example, the incidence of Wolffian duct remnants observed in vaginal sections of 12-month-old control females was 3 in 11 animals; the ductal remnants in one of these was cystic. On the other hand, 6 of 8 DES-100 females, 12 months old, had Wolffian duct remnants associated with their vaginal sections, and in 5 of these animals the ducts were cystic. A common feature observed in mice exposed in utero to DES-100 was excess vaginal cornification (Fig. 1). This increase in keratinization resulted in a thick, irregular epithelial lining. In one case, basal cell hyperplasia of the vagina resulted in the appearance of ‘‘down-growing’’ epithelium with irregular rete pega.

Vaginal adenosis has been found among CD-1 mice treated in utero with DES. Fig. 2 illustrates adenosis in an 11-month-old DES-10 mouse. This change was not seen among the 12- to 16-month-old animals that were the main subject of this report. Glandular elements were noted in the vaginal walls of 14% of the DES-10 mice and 35% of the DES-100 mice (Fig. 3). However, evaluation of serial sagittal sections of these vaginas revealed that the glands were associated with the urethra in these mice. "Female hypospadias," with the urethral opening anterior to the vulva, was usually associated with gland-like structures of urothelial origin, and hyperplasia of urethral glands was often seen.

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In addition to epithelial downgrowths, basal cell hyperplasia, and excess keratinization of the vagina, 5 of the 20 females exposed prenatally to DES (100 μg/kg) had squamous cell changes similar to material described by Dunn (7) as "epidermoid tumors" (Fig. 4). These tumors were localized and not markedly invasive. Three of these animals with tumors also had vaginal concretions, while 2 did not. Vaginal concretions were grossly observed in 7 of 20 DES-100 mice and 3 of 35 DES-10 mice but in no other treatment group.

Of special interest was one DES-10 female with a lesion diagnosed as well-differentiated vaginal adenocarcinoma (Fig. 5). This lesion was found in the vaginal fornix below the surface epithelium. The glands were closely apposed and were usually composed of a single layer of mucin-secreting cells. There was some nuclear pleomorphism, but the most striking feature was invasion of the underlying vaginal tissue. A small lung lesion in the same animal was diagnosed as a primary lung adenocarcinoma.

Cervical Changes. Squamous metaplasia of the cervical epithelium was seen in 25% of the DES-100 mice and in 1% of the controls (Table 2). Other epithelial changes in the treated animals included abnormalities such as mild cellular atypia.

The cervix of the majority of mice exposed gestationally to DES appeared enlarged. This was most common in offspring treated with the higher DES doses (Table 2). Histological evaluation of the uterine cervix demonstrated that both the epithelium and stroma were affected by DES and contributed to the enlargement seen grossly. Although in a group of representative DES-treated females the cross-sectional outer diameter of the cervix was significantly increased over controls (Table 3), the size of the cervical lumen was similar in both groups. The increased cervical size was possibly due to increases in both number and size of epithelial and stromal cells as well as to wide intercellular spaces (cervical edema). Lymphocytes and granulocytes, mostly eosinophils, were usually present, but the number and type of inflammatory cells did not correlate with cervical size.

The stromal contribution to these cervical abnormalities was also suggested by 3 of the lesions seen in the cervical area: a leiomyoma; a leiomyosarcoma; and a stromal cell sarcoma. The latter tumor was composed of anaplastic cells, resembling endocervical stromal cells, which had infiltrated the wall of the uterus, cervix, and vagina (Fig. 6). Moderate mitotic activity, as well as large areas of necrosis, were apparent in the middle of the main mass. The absence of myofibromas on electron microscopic examination of this tumor was used to classify this lesion as a stromal sarcoma rather than a leiomyosarcoma. Metastases to the liver and spleen, as well as to the ovary and oviduct, were observed.

Uterine Changes. Cystic endometrial hyperplasia was noted in all groups of mice in the study. These changes in the uterine endometrium comprised 7% of the control, 21% of the DES-1, and 60% of the DES-100 uteri (Table 2). In some cases, it was so extensive (Fig. 7) that the uterine lumen could not be determined. The epithelial changes in the uterus appeared to be of 2 types, proliferative and metaplastic. One type involved columnar, glandular epithelium and included the areas of endometrial hyperplasia with atypia (Fig. 8), adenomyosis (Fig. 9) in which endometrial glands were seen outside the myometrium, and a uterine adenocarcinoma (Fig. 10).

The second type of uterine epithelial change involved the replacement of columnar with squamous epithelium resulting in extensive areas of squamous metaplasia (Fig. 7). Squamous epithelium was found in some cases lining the entire uterine cavity while, in other cases, it was only in localized areas of the surface or glandular endometrium with columnar epithelium on either side. Squamous metaplasia was not seen in any of the control female offspring.

At the time of sacrifice, the uteri of DES-treated mice were either larger (hyperplastic) or smaller (hypoplastic) than were those of the controls. As with the uterine cervix, the size changes in the DES-exposed uteri appeared to be related to differences in the response of the stromal cells; the largest uteri were comprised of both epithelial and stromal hyperplasia while the smallest contained simple aglandular epithelium with hyalinized endometrial stroma and myometrium. Moreover, as seen in Table 2, stromal tumors were observed in this organ: 2 leiomyomas and one stromal cell sarcoma.

Oviductal and Ovarian Changes. As seen in Table 4, the oviduct of DES-treated mice had a dose-related increase in abnormalities mainly associated with inflammatory changes (salpingitis and pyosalpinx). At the highest dose, a gross structural malformation was observed (Fig. 11b) in which the oviduct was less convoluted than was that of the controls (Fig. 11a) and was located in a similar anatomical position, relative to the ovary, to that seen in the fetal mouse. This abnormal association between the ovary and oviduct often resulted in cystic spaces lined by ciliated epithelium.

Table 4 also shows that prenatal exposure to DES was associated with increases in the frequency and severity of abnormalities in the oviduct. The occurrence of abnormalities in the oviduct was dose-related and was increased in the DES-100 mice compared to the controls. The abnormalities included inflammatory changes and cysts, which were more frequent in the DES-100 group.

Table 5

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Outside diameter</th>
<th>Myometrial width</th>
<th>Luminal measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.7 ± 0.4^a</td>
<td>0.4 ± 0.2</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>DES-100</td>
<td>5.1 ± 0.2^a</td>
<td>1.3 ± 0.2</td>
<td>2.0 ± 2.1</td>
</tr>
</tbody>
</table>

^ Measurement was the longest linear distance from basement membrane to basement membrane across the cervical lumen.
^ Mean ± S.D.
^ Significantly different from controls (p < 0.01).
^ Not significant.

Table 6

<table>
<thead>
<tr>
<th>Abnormalities of the ovary and oviduct in female mice exposed prenatally to DES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females were 12- to 18-month-old offspring of CD-1 mice treated with DES on Days 9 to 16 of gestation. Ovarian abnormalities include cysts, inflammatory changes, and, in DES-100 mice, medullary tubules. Oviductal abnormalities include inflammatory changes, and, in the DES-100 mice, malformations. Details of abnormalities are given in text.</td>
</tr>
</tbody>
</table>

Abnormalities

<table>
<thead>
<tr>
<th>DES dose (μg/kg/day)</th>
<th>Ovarian</th>
<th>Oviductal</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>37/85 (44)^a</td>
<td>1/85 (1)</td>
</tr>
<tr>
<td>0.01</td>
<td>7/15 (47)</td>
<td>1/15 (7)</td>
</tr>
<tr>
<td>1</td>
<td>8/16 (50)</td>
<td>1/15 (7)</td>
</tr>
<tr>
<td>2.5</td>
<td>8/13 (62)</td>
<td>1/13 (6)</td>
</tr>
<tr>
<td>5</td>
<td>9/13 (69)</td>
<td>2/14 (14)</td>
</tr>
<tr>
<td>10</td>
<td>25/30 (83)</td>
<td>5/19 (26)</td>
</tr>
<tr>
<td>100</td>
<td>18/18 (100)</td>
<td>14/18 (75)</td>
</tr>
</tbody>
</table>

^ Numbers in parentheses, percentage.
associated with a dose-related increase in ovarian abnormalities, chiefly cysts; these changes occurred in 44% of the control and 100% of the DES-100 offspring. Although the distinction between types of ovarian cysts is an important one, for the purposes of the present communication, any cystic structure associated with the ovary was classified as an "ovarian cyst" (Fig. 12). One lesion resembling a granulosa cell tumor was observed in a DES-100 at 13 months of age. More severe lesions in the ovaries of treated mice older than those in Table 4 were a gonadal stromal tumor [DES-100, 19 months (Fig. 13)], a granulosa cell tumor [DES-0.01, 21 months (Fig. 14)], and a papillary cystadenoma [DES-0.01, 29 months (Fig. 15)]. An additional ovarian lesion observed only in DES-100 females was a structure resembling medullary tubules resulting in an 'ovotestis-like' appearance in that organ (Fig. 16). In 4 of the 9 animals with smaller cysts in which sufficient ovarian tissue was available for evaluation, medullary tubule-like structures were observed. Similar ovarian lesions were not observed in any aged control mice.

**Genital Changes with Prenatal Exposure to Other Analogous Chemicals.** In another series of experiments, the female offspring of mice treated prenatally with DMS or estradiol (100 μg/kg) were sacrificed at 13 months of age and examined for lesions associated with comparable exposure to DES-100. As shown in Table 5, ovarian cysts were noted in controls and after treatment with DMS or estradiol; 1 of 10 DMS treated females had mild cystic endometrial hyperplasia, and 2 of these mice had focal squamous metaplasia of the endometrium. However, vaginal concretions, excess vaginal keratinization, cervical enlargement, oviductal malformations, or tumors of the genital tract were not observed in females exposed in utero to DMS or estradiol.

**Evaluation of Fetal Ovarian Grafts.** The pathogenesis of the ovarian lesions seen in aged females was studied by grafting ovaries of 8 control and 8 DES-100 fetuses in the kidney capsules of ovariectomized hosts. The results shown in Figs. 17 and 18 demonstrate the developmental capacity of these organs in ectopic sites. Follicular development was similar to that seen in corresponding in situ control organs. In fact, grafts similar to the 3 surviving control grafts were capable of maintaining estrous cycles in ovariectomized females. A striking difference between the 2 types of ovarian grafts, however, was the presence of medullary tubule-like structures in the 2 recovered ovaries derived from DES-exposed fetuses. These results suggest that the medullary structures seen in the ovaries of aged DES-exposed females are related to a direct effect of DES on ovarian development.

**DISCUSSION**

The results of the present communication expand and confirm previous results from our laboratory (21) which clearly demonstrate the association between prenatal exposure to DES in mice and subsequent female genital tract abnormalities, including neoplasia. Abnormalities were observed in the vagina, cervix, uterus, oviduct, and ovary and appeared to be dose related. These genital tract abnormalities made localization of the squamocolumnar junction in the uterine cervix difficult. For example, the location of the squamocolumnar junction in control mice was usually located within the cervix. In DES-100 animals, this junctional zone was difficult to localize because of excess vaginal keratinization and extensive squamous metaplasia occurring in the uterine cervix and horns. Ongoing scanning electron micrographic studies should be useful in this regard (19, 20).

The absence of significant extragenital lesions in mice exposed gestationally to DES suggests that the fetal reproductive tract (especially the Müllerian ducts) is a target for this toxicant. This hypothesis is supported by studies on male offspring from DES-treated pregnancies in which the Müllerian duct remnants in these mice are also maintained and stimulated (24). Prenatal DES treatment may also have an effect on the fetal mesonephric system, since Wolffian duct remnants were observed more often in sections of DES-treated animals than in sections of controls. The cystic, hyperplastic nature of these remnants suggests an effect on their development. In fact, additional studies on DES-treated fetal genital ducts suggest a direct stimulation of the Wolffian as well as of the Müllerian ducts (21). The possible contribution of the Wolffian duct system to an abnormal uterine architecture as well as to cervical hyperplasia and neoplasia merits further evaluation.

Stromal hyperplasia and neoplasia, as well as epithelial atypias and carcinoma of the vagina, uterus cervix, and horns, suggest that both tissue components are targets for DES in the fetus. In fact, in studies of the genital tract of fetuses derived from DES-treated mothers, the stroma shows hyperplastic changes (21). Whether or not the stroma is a primary target for the DES-associated lesions in both the vagina and uterus awaits further study. However, results on estrogenization of the neonatal mouse vagina, with recombination of the stroma and epithelium, have demonstrated that the vaginal stroma may be an important determinant for subsequent developmental abnormalities in that organ (5). The recent report of Cunha and Lung (6) that prostatic morphogenesis is mediated through the interaction of androgen with the fetal stroma raises the possibility that the genital stroma of the fetal mouse is an important site of DES action.

The developmental period during which DES exposure occurs may be critical in determining the long-term effects observed. Thus, DES treatment during the period of genital tract organogenesis may be expected to change the interaction between Müllerian stroma and epithelium as well as between Müllerian stroma and Wolffian stroma (13). Additionally, Müllerian stem cells may be programmed during this period and, depending on later environmental cues, may respond with the production of squamous (epidermoid tumor) or columnar (ad-
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enocarcinoma) cell changes. DES exposures during the neonatal period, on the other hand, may occur after the genetic program for columnar to squamous transformation has been set; thus, lesions induced at this time may differ somewhat from those described in this report.

Adenosis was a rare histological observation in the series of animals described in this report. This was surprising in view of previously published work in neonatally estrogenized mice (10) and the more recently reported work by Plapinger and Bern (28) on perinatal estrogenization. Some obvious possibilities which may explain this discrepancy include: dose of DES; stage of genital tract development during DES exposure; differences in sectioning techniques; or strain, as well as age, of mice. The first possibility must be considered since the administration of DES in studies which resulted in vaginal adenosis (10, 28) was done directly to the neonatal mouse and at much higher doses than those used in this study. Moreover, since the stage of development of the mouse vagina differs when comparing fetal or neonatal tissue (11), the state of vaginal differentiation during DES treatment may account for some of the observed differences in vaginal lesions. Studies in our laboratory on the third possibility4 included transverse sectioning of the vaginal fornix of 12 animals treated with DES-100 (12 to 18 months old). This study failed to uncover any cases of vaginal adenosis. Since Plapinger and Bern (28) have demonstrated vaginal adenosis in perinatally estrogenized mice in at least 4 strains, strain differences alone are unlikely to account for our observations. The last possibility, age of the animals, must be of concern when looking at epithelial lesions in the vagina. In an area of adenosis, the replacement of columnar cells by squamous cells observed in mice4 and previously described in humans (15) raises the possibility that adenosis is not a noticeable feature in older mice because of squamous metaplasia within the lesion. However, previous studies by Forsberg demonstrating adenosis in 11 of 21 mice, 6 months old, (9) and 3 of 3 mice, 13 months old (12), leaves the role of squamous metaplasia in the observed progression of adenosis in mice unresolved. Experiments evaluating transverse sections through the vaginal fornix of 5- to 7-week-old offspring prenatally exposed to DES should permit a more direct comparison between our data and those reported in the literature.

The association of lesions in the genital tract with continuous endogenous estrogen stimulation is an important question. Previous studies (see, e.g., Refs. 3 and 32) have reported that some lesions of the mouse genital tract which follow neonatal treatment with estrogens are dependent on persistent estrogenic stimulation of these tissues (ovary-dependent persistent vaginal cornification). Studies in our laboratory demonstrating that squamous metaplasia of the uterus in prenatally DES-treated mice occurs as a lesion which depends on a second postnatal exposure to estrogens for its manifestation5 suggest that similar considerations may be appropriate for this model. The major ovarian abnormality observed in this study is the association between ovary, oviduct, and uterus. This lesion may represent the female homology of the retained testes described earlier (24) in male mice exposed prenatally to DES. One pole of the ovary is often adherent to the uterus, resulting in cystic areas lined with ciliated epithelium. The epithelium of the oviduct, as well as that of the ovarian surface, was hyperplastic. In addition, a more subtle developmental abnormality involving the fetal genital ducts was seen. The presence of medullary tubule-like structures in the treated ovaries resulted in an "ovestis-like" appearance of the gonad. This abnormality could arise from a prenatal stimulation of the Wolffian duct remnants in the ovary (the rete ovarii) by DES during a critical time of development; support for this hypothesis was derived from the appearance of medullary tubule-like structures in grafts of fetal ovaries explanted in untreated host females. Whether the low incidence of ovarian tumors (0 of 85, controls; 4 of 105, DES, all treatments) seen in these animals is developmentally derived or is the result of secondary factors remains to be determined. However, the data presented clearly establish the ovary as a potential in utero target for the long-term toxic effects of DES.

The actual mechanism for the transplacental toxicity of DES remains unknown (22). The role of altered cell differentiation, perinatal estrogenization, direct carcinogenic action, or some combination of all of these factors in the observed long-term effects of DES on the developing genital system is currently the subject of intense investigation in our laboratory.

Results presented in this report demonstrate that the spectrum of genital tract lesions associated with prenatal exposure to DES (vaginal keratinization, cervical enlargement, cystic endometrial hyperplasia, oviductal malformations, and genital tract tumors) was not induced by similar exposure to estradiol or DMS. In addition, prenatal treatment of mice with estradiol (100 µg/kg), a dose which sterilized similarly treated DES females, had no observable effect on their total reproductive capacity (21, 23). It is possible that this difference is related to potency; higher doses of estradiol may result in genital lesions and sterility. In the outbred (CD-1) mouse used in these studies, however, doses of estradiol or DES greater than 100 µg/kg were not consistent with normal gestation and parturition. These results suggest that relative bioavailability of these compounds to their target sites may influence toxicity (23).

Previous animal studies have demonstrated that, after maternal treatment with DES, the compound enters the fetal compartment and accumulates in the fetal genital tract (31). Moreover, oxidative metabolites of DES have been identified in the mouse during the perinatal period (25). Within the target tissue, DES and some of its metabolites, unlike estradiol, do not bind to an appreciable degree to AFP, the major extracellular estrogen-binding component in the rodent fetus (23). Within the cell, DES metabolites differ widely with regard to the binding to the cytoplasmic receptor for estrogens; DES and estradiol have similar binding characteristics (18).

Whether the difference in binding to AFP can explain the difference in the transplacental toxicity of DES and estradiol is unclear. Eiger (8) reported that, although estradiol stimulated the fetal genital tract (Müllerian vagina) transplacentally in rats, DES and ethynylestradiol were approximately 20 times more potent. Tumorigenesis following estradiol treatment was not described. In a similar experiment, Vannier and Raynaud (33) demonstrated that 17β-estradiol was less effective than was one of its structural analogs, 11-β-methoxy-17β-estradiol, in

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feminizing the male rat genital tract during fetal life. These results were attributed to the lack of binding of 11-β-methoxy-17β-estradiol to rat AFP. Hence, at least in the rat and mouse, differential binding to specific extracellular proteins, and thus the bioavailability of free drug, may play a role in the transplacental toxicity of DES and estradiol. In the human, estrogen binding does not appear to be a major property of AFP (27). Therefore, additional factors such as differences in the rate or extent of conjugation (including sulfation), binding to specific maternal plasma sex steroid-binding proteins, or fetal-placental metabolism should be considered for the human.

The results of prenatal exposure to DES in mice presented in this communication may be relevant for similarly exposed humans. For example, the case of vaginal adenocarcinoma reported here is similar in location and morphology to that reported clinically. Also, the endometrial hyperplasia of the mouse uterus may be important in understanding the upper genital tract abnormalities, including the "T-shaped uterus," observed by hysterosalpingography in humans (14). Finally, recent reports of menstrual irregularity and possible subfertility in women exposed prenatally to DES (4, 14, 29) are similar to that already described for this mouse system (21). These findings, however, are in contrast to those recently reported by Barnes (1) showing no significant differences between the menstrual histories of DES-exposed women and those of unexposed women. Our increasing knowledge of the pharmacology of DES in the mouse, its biochemistry, and the experimental embryology of the mouse influenced by exogenous estrogens suggests that this is an appropriate animal model for studies of the underlying mechanism involved in the transplacental toxicity of DES. Studies from other laboratories on neonatal (2, 3, 7, 10, 28, 32, 34) as well as on prenatal (8, 17, 26, 30) exposures of experimental animals provide additional information in this regard.

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REFERENCES

Abnormalities in Mice Exposed Prenatally to DES

Fig. 1. Heavily keratinized vaginal epithelium; WD, the lumen of a prominent remnant of the Wolffian duct. DES-100. H & E, × 75.

Fig. 2. Adenosis in vaginal fornix represented by benign glandular elements in a normally stratified squamous cell area; note thick keratin layer on adjacent squamous epithelium. DES-10. H & E, × 75.

Fig. 3. Abnormal urethral opening in the vagina; the glandular structures located in the vaginal wall (arrow) are derived from urethral glands. DES-100. H & E, × 25.

Fig. 4. Epidermoid tumor in the vagina with an irregular extension of epithelium into the underlying connective tissue. There is some cellular pleomorphism and a lack of orderly progression from basal to squamous cells. DES-100. H & E, × 75.

Fig. 5. a, submucosal glands in the vaginal fornix. DES-10. H & E, × 40. b, higher magnification of the field shown in a. Glands are composed of a single layer of mucin-secreting cells. There is some nuclear pleomorphism, but the most striking finding is invasion into the underlying tissue. These findings are consistent with well-differentiated adenocarcinoma of the vagina. DES-10. H & E, × 100.

Fig. 6. a, mass in the cervical region of the reproductive tract. Uterine horns protrude off both sides of the mass. The multiple white foci in the liver are metastases from the abdominal mass. DES-5. b, the mass shown in a is composed mostly of anaplastic stromal cells; the lesion was diagnosed as stromal cell sarcoma of the uterine cervix with extension to the uterine horns, ovary, and oviduct. DES-5. H & E, × 75.

Fig. 7. Cystic endometrial hyperplasia with changes which vary from enlarged spaces lined by columnar (top) or flattened (bottom) epithelium. There is squamous metaplasia in the glands in the center of the photomicrograph. DES-100. H & E, × 75.

Fig. 8. Endometrial hyperplasia in this specimen was due to an increase in glands and smooth muscle. Glandular epithelium had a tendency for papillary projections and “piling up” of cells with hyperchromatic nuclei (center) resulting in a diagnosis of atypical endometrial hyperplasia. DES-100. H & E, × 120.

Fig. 9. A single uterine gland at the serosal surface of the uterus (adenomyosis) is outside the myometrium but under the mesothelium. The lumen of the uterus is toward the bottom. DES-5. H & E, × 120.

Fig. 10. Photomicrograph of uterus with adenocarcinoma extending through the uterine wall. DES-100. H & E, × 75.

Fig. 11. a, gross photograph and drawing of an ovary and oviduct (OV) from a control mouse. Note the relationship of the structures and the regular loops of oviduct which were uniform in diameter. × 10. b, gross photograph and drawing of an ovary and oviduct (OV) from a prenatally exposed DES mouse. The oviduct is closely adherent and coiled around the ovary giving a superficial resemblance to testes and epididymis. Also note that the oviduct is irregularly dilated and thin walled. DES-100. × 10.

Fig. 12. Photomicrograph of ovary-oviductal abnormalities in a prenatal DES-treated mouse. The dilated duct contains a papillary structure. There is also evidence of chronic inflammation. DES-100. H & E, × 15.

Fig. 13. Photomicrograph of a pleomorphic gonadal stromal tumor of the ovary. DES-100. H & E, × 120.

Fig. 14. Representative area of a partially luteinized granulosa cell tumor; the tumor occupied the entire ovary. DES-100. H & E, × 120.

Fig. 15. A papilloalveolar pattern is apparent in this cystadenoma of the ovary. There is a thin rim of ovarian tissue around the cyst, lined by ciliated epithelium. DES-100. H & E, × 25.

Fig. 16. Histological section of an ovary with few developing follicles. Most of the area of the organ is composed of light-staining cells arranged in tubule-like structures. DES-100. H & E, × 25.

Fig. 17. Control fetal ovarian tissue grafted under the kidney capsule of an adult female host. Ovarian development has proceeded as in vivo. Note the presence of many follicles in this section. H & E, × 25.

Fig. 18. Treated fetal ovarian tissue grafted under the kidney capsule of a control adult female host. Ovarian development has proceeded as in vivo. Note the presence of “medullary tubule-like structures” (arrow) which were a common ovarian stromal abnormality in treated animals. DES-100. H & E, × 25.
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Long-Term Effects on the Female Mouse Genital Tract Associated with Prenatal Exposure to Diethylstilbestrol

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